Brief Communication

Tumor Suppression by Cell Walls of *Mycobacterium bovis* Attached to Oil Droplets [1]

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SUMMARY—The growth of syngeneic guinea-pig tumor transplants in skin was suppressed if the tumor cells were inoculated together with bacillus Calmette-Guérin (BCG) cell walls attached to oil droplets. Tumor growth was not inhibited if the tumor cells were given together with 1) BCG cell walls, 2) oil droplets, and 3) oil droplets and BCG cell walls prepared so as to prevent attachment of the walls to the oil droplets. Animals in which tumor growth was suppressed acquired systemic tumor immunity.—J Nat Cancer Inst 48: 831–835, 1972.

WE HAVE reported that the intradermal growth of a transplantable, syngeneic guinea-pig hepatocarcinoma in normal guinea pigs is suppressed if the tumor cells are inoculated together with living bacillus Calmette-Guérin (BCG) (*1*, *2*). Heat-killed BCG or extracts of tubercle bacilli did not inhibit tumor growth. For treatment of cancer patients, however, nonliving mycobacterial preparations with tumor-suppressive properties would be preferable to living BCG. We now report that cell walls of BCG attached to oil droplets possess potent tumor-suppressive activity. Preparations of this type are as effective as living BCG in preventing pulmonary tuberculosis in mice and monkeys (*3*, *4*).

MATERIALS AND METHODS

Animals.—Sewall-Wright inbred strain-2 male guinea pigs were obtained from the Laboratory Aids Branch, Division of Research Services, National Institutes of Health (*5*). Skin grafts between members of the strain are not rejected (*6*). Guinea pigs were grouped 6 per cage and fed Wayne guinea pig chow daily and kale 3 times a week.

Tumors.—We have described the induction of primary hepatomas in strain-2 guinea pigs fed the water-soluble carcinogen diethylnitrosamine (*7*) and the antigenic and biologic properties of the transplantable tumors derived from the primary hepatomas (*8*, *9*). A transplantable hepatoma designated tumor line 10 (seventh to eleventh transplant generations) was used. Tumor line 10 is a hepatocellular carcinoma converted to ascites form. Intradermal inoculation of 10^5 ascites line-10 tumor cells leads to progressive intradermal growth and metastases to the regional lymph nodes. Animals

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die about 60 days after intradermal inoculation of 1.5×10^6 tumor cells (*2*).

Preparation of BCG cell walls.—Cell walls of the Glaxo strain of BCG were prepared as in (*10*). Mycobacteria were grown in Sauton's medium at the Lederle Laboratories (Pearl River, N.Y.). After 3 weeks in culture, the bacteria were disrupted in the Sorvall refrigerated pressure cell at 35,000 pounds per square inch. Cell walls were obtained by centrifugation of the mixture at 20,000 × *g* for 1 hour. The pellet was resuspended in distilled water and washed several times until only cell walls were visible under the electron microscope.

Preparation of BCG cell walls attached to oil droplets.—Freeze-dried cell walls (25 mg) were placed in a 15-ml tissue grinder equipped with a Teflon pestle (Scientific Glass Apparatus Co., Inc., Bloomfield, N.J.). A light mineral oil (0.12 ml), Drakeol 6 VR (Pennsylvania Refining Co., Butler, Penna.), was added to the cell walls. This mixture was ground to a smooth paste; the pestle was rotated at 800 rpm. Ten ml 0.85% NaCl containing 0.2% Tween 80 (polyoxyethylene derivative of sorbitan mono-oleate, Atlas Powder Co., Wilmington, Del.) was added to the paste, and grinding was continued until a well-dispersed oil-in-water emulsion was obtained. The emulsion was poured into a sterile container and 6.7 ml Tween-saline diluent was added to the grinding tube. Grinding was continued for 2–3 minutes. The 2 emulsions were combined and ground for an additional 2–3 minutes. This emulsion containing 1.5 mg cell walls per ml was heated at 65°C for 30 minutes in a water bath. Examination of the product with the light microscope showed spherical oil droplets varying from $<1\ \mu$ to $>15\ \mu$; discrete particles appeared to be attached to the surface of the oil droplets. Photographs of similar emulsions are in (*11*).

Preparation of oil droplets alone, cell walls alone, and cell walls not attached to oil droplets.—Oil droplets alone: This preparation was made in the same way as the BCG cell walls attached to oil droplets except for the omission of the cell walls. Under the light microscope this product showed clear spherical oil droplets varying from $<1\ \mu$ to $>15\ \mu$. Cell walls alone: The method was the same as for the BCG cell walls attached to oil droplets except for the omission of the oil. Under the light microscope this product showed cell wall fragments varying from 2–5 μ. Cell walls not attached to oil droplets: In this preparation, the oil in water emulsion was made first and the cell walls were added without further grinding. The cell walls were dispersed in the emulsion by gentle mixing. Under the light microscope this product showed clear spherical oil droplets of varying size and aggregated cell walls that remained unattached to oil droplets.

Preparation of tumor cell mixtures for inoculation.—Ascites tumor cells, obtained as described in (*12*), were washed 3 times in medium 199 without antibiotics. Equal volumes of ascites tumor cells and the appropriate emulsion or cell wall preparation were mixed at room temperature in 13 × 100 mm siliconized, sterile, glass test tubes; they were incubated at 37°C for 10 minutes, and then 0.1 ml was injected intradermally into unimmunized guinea pigs.

Measurement of delayed skin reactions and papules.—Delayed skin reactions were measured 24 hours after injection of the mixtures. Papules were measured at several intervals after injection. Two diameters of the skin reaction or papule perpendicular to one another were measured. The two diameters were used to calculate the square of the average radius of the skin reaction or papule. In experiments in which several animals were challenged with portions of the same tumor-cell mycobacterial antigen mixture, the square of the average radius for each lesion produced by the same number of tumor cells was calculated. Results were expressed as the mean of the square ± the standard error of the mean.

Each experimental group contained 3 guinea pigs. At each time point, the results represent the mean value of 3 animals. Each animal received a single test mixture.

RESULTS

In preliminary experiments, oil-treated cell walls of BCG were injected intradermally into guinea pigs immunized with BCG and into unimmunized guinea pigs. The following day, typical delayed cutaneous-hypersensitivity reactions were seen only in guinea pigs immunized to BCG. Three days later, in immunized and unimmunized guinea pigs an inflammatory nodule was at the injection site of

oil-treated cell walls of BCG. This nodule persisted for 3 weeks. A small scar was left at the site of the healed nodule. This experiment indicated that oil-treated cell walls of BCG contained mycobacterial antigens and, like living BCG, produced a chronic inflammatory nodule which healed in 3–4 weeks.

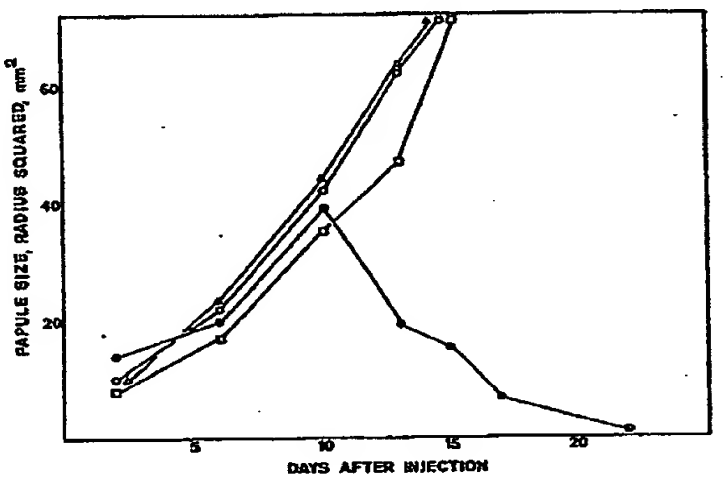
The possible tumor-suppressive properties of oil-treated cell walls were evaluated in the next experiment. Group 1 received an intradermal injection containing oil-treated cell walls (75 μg) and 1.5×10^6 living line-10 tumor cells. Group 2 was given an intradermal injection containing oil droplets without cell walls and 1.5×10^6 living line-10 tumor cells. Group 3 received an intradermal injection containing 1.5×10^6 living line-10 cells diluted with 0.85% saline containing 0.2% Tween. Group 4 was inoculated intradermally with 1.5×10^6 living line-10 tumor cells diluted with 0.85% saline without Tween. The results of the experiment are illustrated in text-figure 1. Oil-treated cell walls completely suppressed tumor growth. Oil droplets without cell walls or saline containing Tween did not inhibit tumor growth.

The following experiment was designed to determine whether bacterial cell walls without oil droplets would inhibit tumor growth and whether the cell walls had to be attached to the oil droplets for tumors to be suppressed. Group 1 received an intradermal injection of 75 μg oil-treated cell walls + 1.5×10^6 living line-10 tumor cells. Group 2 was given 75 μg cell walls without oil droplets + 1.5×10^6 living line-10 tumor cells. Group 3 received 75 μg cell walls not attached to oil droplets + 1.5×10^6 line-10 cells. Group 4 was treated with 1.5×10^6 line-10 cells diluted with saline containing Tween. The results of this experiment (text-fig. 2) indicate that cell walls alone and cell walls in the presence of, but not attached to, oil droplets were ineffective in tumor inhibition.

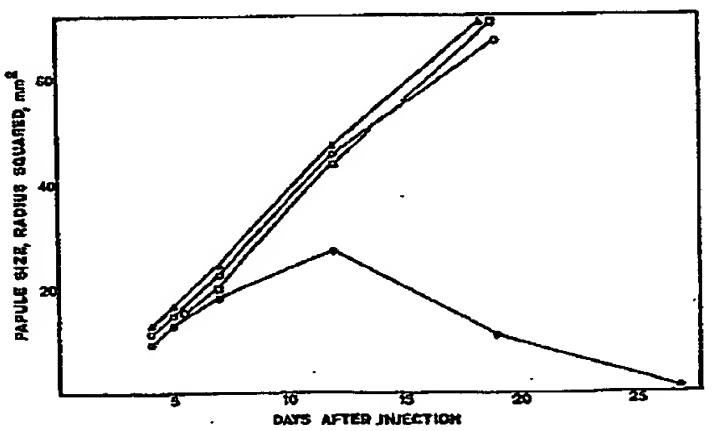
To test whether guinea pigs treated with a mixture of oil-treated cell walls of BCG and tumor cells had developed systemic tumor immunity, guinea pigs received intradermal injections of 10^6 living line-10 tumor cells. The challenge dose was inoculated contralateral to the immunization site. The presence of delayed cutaneous hypersensitivity reactions to tumor cells and prevention of tumor growth (table 1) indicate that guinea pigs treated with vaccine had developed systemic tumor immunity.

DISCUSSION

These experiments demonstrate that a nonliving mycobacterial preparation can completely suppress tumor growth. The tumor-suppressive property of oil-treated cell walls is in contrast to the previously



TEXT-FIGURE 1.—Suppression of tumor growth by oil-treated cell wall of BCG. ●: Cell walls attached to oil droplets + 1.5×10^6 line-10 tumor cells. ○: Oil droplets + 1.5×10^6 line-10 tumor cells. □: 0.85% saline containing 0.2% Tween 80 + 1.5×10^6 line-10 tumor cells. △: 0.85% saline + 1.5×10^6 line-10 tumor cells



TEXT-FIGURE 2.—Suppression of tumor growth by oil-treated cell walls of BCG. ●: Cell walls attached to oil droplets + 1.5×10^6 line-10 tumor cells. ○: Cell walls + 1.5×10^6 line-10 tumor cells. □: 0.85% saline containing 0.2% Tween 80 + 1.5×10^6 line-10 tumor cells. △: Cell walls not attached to oil droplets + 1.5×10^6 line-10 tumor cells.

TABLE 1.—Tumor immunity after intradermal injection of oil-treated mycobacterial cell walls and living tumor cells

Treatment	Delayed cutaneous hypersensitivity reactions at 24 hr—radius squared, mm²		Tumor incidence at 10 days after challenge with 10^6 tumor cells	
	Expt. 1*	Expt. 2	Expt. 1	Expt. 2
Oil-treated cell walls and living tumor cells	27 ± 2	Not done	0/3	0/3
None	7 ± 1	Not done	6/6	5/5

*In expt. 1, guinea pigs were challenged the 28th day after immunization and in expt. 2 the 22d day after immunization. Immunized animals were free of tumor at the time of this report, 2 months after challenge. Tumors in the unimmunized group grew progressively.

reported (*2*, *13*) lack of tumor inhibition in guinea pigs not immunized to BCG when other nonliving mycobacterial antigen preparations were used (cell walls alone, purified protein derivative, heat-killed BCG, crude tuberculoprotein). In immunized guinea pigs, tumor may be partially suppressed by these nonliving antigens. There have been reports that nonliving preparations or fractions of mycobacteria are effective in tumor inhibition (*14*). The experimental model described in this report offers the possibility of comparing the tumor-inhibiting capacity of nonliving preparations of mycobacteria.

Injection of oil-treated cell walls mixed with living tumor cells leads to the development of tumor immunity. Recent studies of tumor immunity produced by injection of mycobacterial antigen-tumor cell mixtures indicate that differences exist in the intensity of tumor immunity produced by different mycobacterial antigen preparations (*15*). For example, intradermal immunization with complete Freund's adjuvant and tumor cells was not as effective as intradermal immunization with living BCG and tumor cells.

We do not know why BCG cell walls attached to oil droplets can suppress tumor growth. If the mechanism of tumor suppression by oil-treated BCG cell walls is similar to that obtained with living BCG, this means that in some unknown fashion the oil droplets make it possible for the recipient to respond immunologically to BCG cell-wall antigens by the production of specifically sensitized lymphocytes. Interaction of these lymphocytes and BCG antigens in the presence of tumor cells causes the accumulation of nonlymphoid cells responsible for tumor cell death.

This work may provide a simple, rapid model for determining the components of the tubercle bacillus required for tumor suppression and indicates that a nonliving mycobacterial antigen preparation may be clinically useful for inhibiting tumor growth.

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